Madich A, Richardson GD, Jahoda CAB. [Contribution of GFP expressing Dermal Papillae cells to the formation of chimeric embryos and their survival in uterine environment](#). *British Biotechnological Journal* 2016, 12(2), 23393.

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DOI link to article:

<http://dx.doi.org/10.9734/BBJ/2016/23393>

Date deposited:

03/03/2016

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Contribution of GFP Expressing Dermal Papillae Cells to the Formation of Chimeric Embryos and their Survival in Uterine Environment

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AM, GDR and CABJ designed, performed and managed the analyses of the study. Author AM managed the literature searches, wrote the protocol, performed the statistical analysis, wrote the first draft of the manuscript, and did all correction mentioned by the reviewers. Author CABJ coordinated and supervised the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2016/23393

Editor(s):

(1) Shinya Shimada, Director, Kumamoto General Hospital, Japan Community Health Care Organization (JCHO), Japan.

Reviewers:

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(3) Luis Ricardo Martinhao Souto, Universidade de Marília, Brazil.

Complete Peer review History: <http://sciencedomain.org/review-history/13311>

Original Research Article

Received 29th November 2015

Accepted 20th January 2016

Published 15th February 2016

ABSTRACT

Aims: This is an innovative cell-based research project focused on the hair follicle dermal papillae (DP) as a source of key-element cells for regeneration of hair growth. DP is major dermal compartment which has a role in hair formation at embryogenesis and is able to differentiate down an endothelial lineage for *in vitro* functional assays. In the present study, we have tested the potential of DP cells from the lower end bulb region of hair follicles for multilineage differentiation *in vivo*. We have postulated that DP cells' epigenetics can be changed under the influence of embryonic microenvironment when they are injected into early embryos which would demonstrate plasticity, regenerative and inductive properties of hair follicle dermal cells.

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Study Design: Pilot research.

Place and Duration of Study: School of Biological and Biomedical Sciences, University of Durham, the UK, between February 2007 and December 2009.

Methodology: To identify the capacity of functional contribution to organogenesis we microinjected various numbers of Green Fluorescence Protein (GFP)-expressing Dermal Papillae cells (GFP-DP cells) into mice blastocysts and transferred embryos to the foster mothers for the generation of chimeric fetuses.

Results: GFP-expressing cells were detected in 3% of the epiblast egg cylinders formed from microinjected blastocysts cultured in absence or presence of mouse embryonic fibroblasts (MEFs). Surgical transfer of microinjected blastocysts resulted in 4.5% of fetuses developing fully. GFP- DP cell lineages were detected in tissue samples under fluorescence and confirmed immunohistochemically. Our finding displayed subcultural localization of GFP as a proof of chimaeric tissue from unborn pups carrying DP cell lineages which had multiplied in bone marrow, parenchyma and connective tissue, brain and hair bulbs.

Conclusion: The results confirm the capability of a small population of DP cells to convert into embryonic stem-like cells, multiply and contribute to organs and tissue. This makes them a reasonable source for regenerative studies and replacement therapy.

Keywords: Dermal papillae cells; blastocyst; microinjection; differentiation; pluripotency.

1. INTRODUCTION

The capability of epigenetic reprogramming somatic cells to convert into embryonic stem- like cells attracts much attention because of the potential for customized transplantation therapy, as cellular derivatives of reprogrammed cells will not be rejected [1-3]. The DNA methylation, gene expression and chromatin state of such induced reprogrammed cells are similar to ES cells [4-6]. Innovative cell-based therapies have focused on the dermal papillae cells (DPCs) as a way to grow new hair in previously bald areas. Current research in this area tended towards the use of developmental biology-based tissue engineering approaches to try to create a skin equivalent that will produce hair follicles when grafted *in vivo* [7,8].

The dermal papilla is the major dermal compartment in hair formation during embryonic morphogenesis and hair cycling after birth [9-11]. Dermal cells in the lower end bulb region of hair follicles are highly active and able to differentiate down an endothelial lineage and they exhibit similar functional characteristics to endothelial cells in *in vitro* functional assays. Data suggest that the majority of DP cells in primary cultures have the ability to function as endothelial cells when exposed to angiogenic signals *in vitro* [12-14]. The identification of dermal papillae cells' properties is previously clearly documented *in vivo* and *in vitro*, confirmed *in situ* and detected by transmission electronic microscopy [15,16].

Studies suggest dermal papillae (DP) cells are a source of key element-cells for regenerating hair growth. Because DPs are located at the base of the hair follicle, which is a unique tissue surrounded by epithelial matrix cells, they provide regulating factors and nutrients to support the proliferation and differentiation of the epithelial matrix cells in hair cycle progression and also in follicle formation in embryonic skin [17-19]. Cells from DPs possess several characteristics that distinguish them as adipose-derived MSCs (mesenchymal stromal stem cells), including their capacity to self-renew indefinitely and differentiate into cells of other follicular or interfollicular regions [20].

Previous basic research revealed that dermal macroenvironment is important in the maintenance of bulb cell population and hair follicle growth [21,22]. Based on findings that demonstrate the plasticity as well as regenerative and inductive properties of hair follicle dermal cells [23,24], we have postulated that their epigenetic can be changed under the influence of an embryonic microenvironment when DPCs are injected into the cavity of an expanded blastocyst. They may multiply and contribute to organs and tissue of mosaic animal models after transferring microinjected embryos to foster mothers.

In comparison to the newly verified potential of a few proteins to undergo direct reprogramming, the microinjection technique is a useful tool to determine the unique capability of early pre-implantation embryos to return from a status of

multipotent cells back to embryonic by "collaboration" with embryonic blastomeres. This is because of the earlier embryos' possession of secret transcription factors that are the main key of cellular totipotency and pluripotency [21]. Previous research detected that the cells derived from mouse embryonic fibroblasts and injected into blastocysts are able to promote the epigenetic reprogramming of their genome to an embryonic state [4]. Papillae cells derived from hair follicles demonstrate a lower proliferative capacity compared to dermal fibroblasts but can be serially cultured for a long period without losing their hair-inductive potency [20,25].

Because of our interest in testing DP cells' pluripotency under blastocyst control, we examined the development of microinjected embryos *in vitro* in the absence and presence of MEFs. Our results indicated that a part of DP cells is able to rapidly differentiate into embryonic-like cells contributing to epiblast cylinders. Furthermore, we demonstrated that mosaic fetal tissue possesses some chimerism resulting from DP cells' microinjection. Because microinjected DP cells gave a start to multilineage differentiation, the pluripotent property of these cells is evidenced under certain conditions. Our results revealed that DP cells are not only able to produce skin type cells but, examined in development *in vivo*, they showed the ability to differentiate into several somatic cell types of the embryo proper, suggesting that DP cells can be reprogrammed to assume a state analogous to the embryonic stem-like cells. We have shown that cellular GFP markers from GFP-expressing DP cells were found in various organs and tissues of the fetuses derived from the microinjected blastocysts as a result of fusion events between DP cells and original embryonic blastomeres.

2. MATERIALS AND METHODS

2.1 Cell Isolation and Maintenance

The most widely used method to isolate DP population is surgical microdissection, which has been successfully established in rodent vibrissae follicles [15,22]. GFP-expressing DPCs were obtained from the hair bulbs of mutant transgenic mice line C57BL/6 with an "enhanced" GFP cDNA under the control of a specific promoter (human ubiquitin C) which makes all tissue express GFP. We used 18 animals, 24-72 weeks old, both males and females as GFP-DPCs donors.

Briefly, the vibrissae were pulled forward and an arching incision was made under the eye, from mouth up to the bridge of the nose. The incision in the vibrissae area was dissected to separate the outer and fur layers from the underlying surrounding connective tissue. The largest hair follicles were selected to separate their proximal-most tips. After removal they were placed in individual drops of DMEM (Dulbecco Modified Eagle' Media) with antibiotics (0.1%) and antifungal (0.2%). Specific dissection technique was used to minimize contamination of the DP cell population with other mesenchymal and epithelial cells and matrix [19]. After isolation of collagen capsules, the papillae were then transferred to a 1 ml well in a four-well plate that contained 20% FBS in DMEM. A fine pointed needle was used to separate the outer dermal sheath layer and part the epidermal matrix component from around dermal papillae in each end bulb under a microscope x10. Each well contained 4-5 papillae to increase the chance that they would adhere for a few days. Papillae were cultured in a medium known to induce adipocyte and osteocyte differentiation [20]. The outgrowth of DPCs was observed with flattened and polygonal morphology. Cells were propagated by changing media until spheres formed around the papillae. When an appropriate confluence was reached the cells were transferred to a larger culture flask into ESC media based on DMEM containing non-essential amino acid (NEAA), β -mercaptoethanol and 20% FCS. They were further cultured for over 1 month until a homogenous cell population was created, then dispersed by repeated pipetting and seeded for use as a single cell suspension at various passages (p 3-4 or p 6-8) for blastocyst microinjection.

2.2 Embryo Handling and Blastocyst Microinjection for *in vitro* and *in vivo* Studies

Day 3.5 blastocysts were flushed from the uteri of pregnant females from inbred mice line BALB/c (C57BL/6j x CBA/j) F1 that had mated with the same males in natural oestrous (*in vitro* trials) or after hormonal induction of superovulation (*in vivo* studies) to get a bigger number of injectable embryos. The uterine horns were flushed with M2 medium while the embryos were washed briefly in a few M2 medium drops and kept in M16 medium droplets at 5% CO₂ and 38°C.

Microinjections were performed with Eppendorf Manipulation system in M2 medium, 5-10 or

10-15 DP cells into each fully-expanded blastocyst. Microinjected embryos were placed into KSOM medium drop at 5% CO₂ and 38°C to revive and re-expand after a collapse caused by a micropipette penetration, till all microinjection procedures were complete.

2.3 Long-term Embryo Culture and Generation of Novel ESC Lines from Microinjected Blastocysts

Mouse Embryonic Fibroblasts (MEFs) were prepared from 13 to 14 day prenatal fetuses and used to get a feeder MEF layer for novel ESC line derivation. MEFs were treated for mitotic inactivation through exposure in culture to 1 µg/mL solution of mitomycin C in DMEM, plus 10% FBS supplemented with 2 mM L-glutamine and antibiotic, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino-acids (NEAA), and 0.2 mM β-mercaptoethanol (Feeder medium), for 2 hours. After washes (x3 times) in Dulbecco's phosphate buffer solution (DPBS) feeder cells were plated at a density of 5 x 10⁴ cells per cm² and used at passage 3 (p3).

Blastocysts were microinjected with 5-10 GFP-DPCs at passage 6-8 (p6-8), cultured for 2 hours and observed under a fluorescent microscope to ensure GFP-DPCs were incorporated into the blastocysts' cavities. The embryos recognized as bearing GFP-positive DP cells were placed into groups of 20-25 embryos or seeded individually for long-term culture in DMEM media (Gibco) supplemented with 2.2 g/L NaHCO₃, 2 mM L-glutamine (Sigma), 0.1 mM β-mercaptoethanol (Sigma), 20% heat-inactivated FCS (HyClone). 4x10³ U/mL LIF was added at day 5 and on every proceeding second day till substantial epiblast outgrowths had formed. Microinjected embryos were cultured in groups for 12 days and the saved GFP-expressing DP cells and dynamic changes in embryonic morphology were monitored under a fluorescent microscope.

Individual culture of microinjected embryos suggested the application of MEFs. The epiblast egg cylinders were detached from the trophoblast monolayer outgrowth using a glass pipette tip, transferred via a few 20 µL drops of trypsin solution (disaggregation), DMEM plus 20% FCS (trypsin inactivation) and DPBS (for rinsing) to separate the epiblast into the small cell clumps. These were then incubated for the next 48 hours. The newly formed epiblast cell clumps were detached in the same way and transferred into fresh wells of inactivated MEFs.

When epiblast outgrowths were formed the cycle was repeated. 4-5 days after the last picking and seeding the feeder wells containing primary ESC colonies were observed under a fluorescent microscope to assess GFP expression of multiplied GFP-DPCs.

2.4 Embryo Transfer

Microinjected blastocysts were surgically transferred into uteri of pseudo-pregnant CD-1 Albino females, 12-15 embryos unilaterally or 16-20 bilaterally per mouse. The pregnant females were sacrificed when they reached E16.5, E18.5 and E20.5. Foetuses were removed by caesarean section to evaluate the chimerism level and GFP expression of DP cell lineage.

2.5 Immunofluorescent Analysis of GFP Expression

Rinsed from blood, the fetuses were fixed in 4% paraformaldehyde solution in PBS at 4°C for 1 hour and 20% sucrose in PBS at room temperature (RT) overnight, dissected to three parts, then placed in embedding chambers with O.C.T. 4583 (Tissue-Tek, Sakura) and slowly frozen. Parts of the fetuses were sectioned vertically using Leica CM3050S cryostat to a thickness of 8µm. Serial 8 µm sections were taken as tissue sample pairs with 24 µm intervals between every third pair, placed on microscope adhesive slides and stored in air-light moisture protected chambers at -80°C. Each pair of slides were screened either under a fluorescent microscope or stained with GFP-antibodies to catch GFP- DP cell lineages in respect to all organ and skin structures. Frozen sections were washed in 0.2% Tween-20 in PBS (x 3), acetylated and dehydrated through graded ethanol series with using Haematoxylin-eosin staining. Sections were cover slipped with DPX mounting media (Maviol) and 2% DAPI in PBS and visualized in real time to recognise GFP expression.

Fluorescent microscopy was applied to identify GFP using a Carl Zeiss Imager M1, AXIO HBO 100 and a laser excitation wavelength of 488 nm. GFP fluorescence was recorded using band pass 505-550 nm filters and 56-57.8 µm pinhole diameters. Red spectrum wavelengths were excited with 543 nm laser and recorded using long pass 560 nm filters and 62.0-63.0 µm pinhole diameters. GFP expression was detected using enhanced chemiluminescence and captured by the FluorChem 8900 Imaging

system (Alpha Innotech). GFP-positive and GFP-negative control tissue samples had been prepared previously and were used to compare GFP expression intensity.

2.6 Immunohistochemical Analysis / Staining with GFP Antibody

The slides were washed in 0.3% Triton x 100 in PBS to increase the cell membranes' permeability, then rinsed in PBS (2 x 5 min) using a shaker. The rest of the liquid was removed, and a block goat serum diluted 1:10 was used (30 min incubation at RT in wet chamber). Primary GFP rabbit polyclonal antibody ab290 (Abcam) was added in dilution 1:2000 in PBS, and slides were incubated for 1 hour at 37°C under a paraffin film. After washing in PBS (3x5 min) excess liquid was removed and a secondary goat antibody 66s was added in dilution 1:500 with following incubation period for 1 hour at RT in air-light preserved and wet chambers. The slides were carefully and thoroughly washed in PBS (3x5 min) with a shaker. The incubation with 3% hydrogen peroxide (Sigma) for 7 min at RT was aimed to inactivate cells and tissues that have auto fluorescence. Solution Strept AB Complex (Streptavidin HRP Conjugate, HRP Duet Mouse/Rabbit, DK-2600, DAKO) in dilution 1:250 in PBS was used to fix the secondary antibody, with an incubation time of 30 min at RT and a short 5 min rinse after that. To finish the glass slides were incubated for 5 min with toxic solution (Diaminobenzidine, D4293-50, Sigma, dissolved in distilled water). Finally the slides were rinsed in distilled water, acetylated and dehydrated through graded ethanol series using Haematoxylin solution (Fluka). After 5 min washing in Histoclear solution the sections were cover slipped with DPX mounting media (Maviol, R 1340, Agar Scientific).

3. RESULTS

3.1 Establishment of Dermal Papillae Cell Culture

As previously noted, DP cells were isolated from C57BL/6 mice with an "enhanced" GFP cDNA. This mice strain demonstrates a high proportion of GFP-expressing cell populations in epidermis, bone marrow, thymus, spleen, uterus and peripheral blood. The hair follicle bulbs are more easily distinguished in the fat and connection tissues of the vibrissae area because of black phenotype. More prospective follicles located in

the dorsal area, and young animals are preferable donors for primary DPC culture [16]. Vibrissae end follicle bulbs were dissected from the mistrial pad of GFP-transgenic adult mice, and primary cell cultures were established from the papillae explants. Primary follicle dermal explants formed spheres in culture on laminin coating substrate, as described [18]. The cultured papillae cells spread slowly, initially as a monolayer, and eventually formed multi-layered arrays of fibroblast-like cells. At the edges of the expansion, the cell colonies were larger and flattened and showed a tendency to form clumps. Produced DP cell lines displayed the extended proliferative capabilities and at 3-4 passages were ready for microinjection. Most of the visualized DP cells, about 70-80%, were recognized as GFP-positive expressing (fluorescent microscopy applied), typical size 5-15 µm. Employing a laser excitation wavelength of 488 nm showed high fluorescence in round DP cells. We suggest dead or elongated cells that didn't restore their round shape after trypsin treatment (for disruption of cell monolayer) don't diffuse fluorescence. The round DP cells are actively dividing cells that rounded up in the process of dividing and they were chosen for cell microinjection.

3.2 Assessment Ability of Microinjected Blastocysts to Survive *in vitro*

To identify ability of hair follicle cells to function in a similar manner to embryonic stem cells during development, we injected GFP expressing DPCs into D3.5 blastocysts harvested from mice that did not carry the GFP gene. Blastocysts (n=164) deposited into a drop of the M2 medium containing a suspension of GFP-DP cells were injected with 5-10 DP cells per embryo through the trophoblastic layer into blastocoels (Fig. 1).

GFP protein expression was used as a specific reporter for visualizing successful cell microinjection. In a few cases, due to a high pressure in the cavities of some over expanded blastocysts, DPCs were recognized as having gone out into perivitelline space. This led to the loss of injected cells at blastocyst hatching. Microinjected embryos were analyzed for the presence of GFP-DP cells under fluorescent microscopy with a FITC filter set (BP 450-512 nm, DM 512 nm, BF 515 nm). 12 hours later GFP-DP cells were detected in 43.3% blastocysts (71 out 164 injected), in 41.5% at 24 hours, in 36.6% at 48 hours, and in 35.4% (36 out of 104) at 72 hours. GFP protein expression

rate remained stable for the next 24 hours (Fig. 2). Blastocysts attached to the bottom of a Petri dish and developed spontaneously. A culture media was changed just once on the 4th day in order to avoid some morphological damages. The 5th day was noted as a final day for the formation of epiblast outgrowths from hatched embryos.

Fig. 1. Microinjection of GFP-expressing Dermal papillae cells (DPCs) into mice blastocysts. Panel shows a photomicrograph of DPCs cultured before microinjection (A). Microinjection of GFP-DPCs into a blastocyst step-by-step (B, C, D). Panel E shows DP cell culture consisting from GFP-positive and GFP-negative cells (dead or elongated cells that didn't restore round shape after detachment from laminin coating surface). Fluorescent photomicrographs show corresponding GFP-expressing cells in re-expanded and half-hatched blastocysts the day after microinjection (F, G, H). Panels I and J demonstrate injected blastocysts with DPCs corresponding to GFP expression as single and multiple green signals. Some injected cells dissociated and distributed between embryo primary blastomeres. They are not visible in transmitted light (K) but easy distinguishable by fluorescence (L).

At 120 hours the rate of GFP expression in cultured blastocysts decreased to 23.8%. Some epiblastic clumps spontaneously detached from the trophoblastic layer and swam in the upper layer of culture media as hardly-visible flakes. On days 6 and 7 GFP-expressing cells were recognized in 17.1% and 16.5% cell epiblasts, accordingly. They continued to express a low intensity fluorescent signal on days 8 and 9, localized in 13.4% and 9.7% cell epiblasts.

During culture GFP-DP cells did not show a greater number than when initially injected but at day 12 a weak fluorescence was still visible, detected in 5 cell spheres (Table 1).

Fig. 2. Fluorescent light images of hatched blastocysts microinjected with 5-10 injected DPCs which carry a green fluorescent protein (GFP) marker and cultured during 12 days without feeder cells. Photomicrographs show microinjected blastocysts cultured in vitro for 48 hours (A, B) and 96 hours (C), on day 5 (D) and day 6 (E, F) after passing. Panels G and H show residual GFP markers that were still visible in very small cellular compartment on days 9 and 12 after passing.

To extend our *in vitro* trials we checked the hypothesis that DP cells would better incorporate into blastocysts if the microinjected embryos were seeded and cultured on the inactivated MEFs. The study aimed to derive novel ES cell lines and to this end we microinjected 85 blastocysts with 5-10 GFP-DP cells. They were evaluated as the embryos with well-identified GFP protein expression. During culturing the hatched blastocysts formed epiblast outgrowths suitable for picking up within the next 2 days and which reflected visibly bright GFP signal.

Table 1. Summary of *in vitro* and *in vivo* survival of GFP-dermal papillae cells microinjected into blastocysts

Methods	Embryos handling condition	No. microinjected blastocysts	GFP-DP cells number and passage	Value of GFP cDNA expression in embryos / fetuses after cell microinjection at	
				Start point	The end of culturing
<i>In vitro</i> Long term culture for 12 days. In absence of feeder -MEFs	Flushed from females in natural oestrus	164	5-10 cells p6-8	43.3%	3%
<i>In vitro</i> Targeted to novel ESCs lineages generation. In presence of MEFs		85	5-10 cells p6-8	47%	Wasn't detected
<i>In vivo</i> development. Experimental group	Flushed form females in induced oestrus	598 microinjected 534 transferred to 28 foster mothers	10-15 cells p3-4	89.3%	Pregnancy rate - 35.7% Embryo recovery rate 4.5%
<i>In vivo</i> development. Control group		Intact 50 Transferred to 3 foster mothers	No cells/ No micro injection		Pregnancy rate 100% Embryo recovery rate 30%

Abbreviations: GFP, Green fluorescence protein; MEFs, Mouse embryonic fibroblasts; DPCs, Dermal papillae cells

We detected GFP-DP cells contributed to 47% of the epiblast cylinders (40 out 85). The rest of the blastocysts examined failed to hatch or had no visible GFP-expression. After first picking and cell disaggregation, 22.2% of the epiblast tips showed weak visible fluorescence, nearly ¼ of the initial rate, but after second picking only individual epiblast tips were observed as GFP-expressing (Fig. 3).

3.3 Assessment of *in vivo* Chimerism in Foetuses and Survival of DP Cells in Uterine Environment

DP cells were tested for the ability to contribute to embryonic architecture and generate chimeras by the microinjection of GFP-DP cells into blastocysts (n=555) and into compacted morulae that reached blastocyst stage in KSOM media for 2 hours (n=43). In total, 598 blastocysts were successfully injected with 10-15 GFP-DPCs (p3-4) and then 534 embryos (89.3%) were transferred to the 28 CD-1 foster mothers after fluorescent estimation confirming DP cells' incorporation. To compare the main indexes such as a pregnancy level and embryo recovery rate 50 intact blastocysts were transferred to the 3 foster females.

The recipient females were sacrificed at the 13th, 15th and 17th days that correlate with day of embryo development E16.5, E18.5 and E20.5. Pregnancy was detected in 35.7% females (10 out 28) in the experimental group. In total 24 sites (4.5% embryo recovery rate, 24 out 534) were found at caesarean sectioning and 18 out 24 were considered as full-time developed fetuses. Care was taken to calculate the number of non pregnant recipients, encapsulated embryos, partially resorbed fetuses (placenta rests) and females with arrested development. All 3 females in the control group became pregnant (30% embryo recovery rate, 15 out 50) versus 4.5% in experimental group ($P=.01$).

Fluorescent microscopy was employed to detect tissue-located GFP-expressive cell lineages. Two full-time developed fetuses were suspected of significant incorporation of GFP⁺ cells that were visible at resolution x10 (Fig. 4). Both fetuses showed detectable GFP signal in the heads and the bodies when cryosectioned and examined under an immunofluorescent microscope and this was then confirmed with Haematoxylin and Eosin staining.

Fig. 3. Phase-contrast photomicrographs show progressive *in vitro* embryo development following blastocysts hatching and plating (A, B) and primary egg epiblast cylinders forming at day 5-6 after microinjection (C). Panel D shows a decreasing cell mass in epiblast tips after picking and passing for next 48 hours. Phase-contrast microscopy displays a primary cell nest formed following cell dissociation of epiblast tips and passing them on to a feeder MEFs layer 3 days later (E, F). Photomicrographs demonstrate cell nests of different morphology raised after the second (G and H) and third (I and J) circles of cell dissociation.

Our finding suggested that GFP⁺ cell derivatives were likely to be present in epiblast-derived lineages and this was confirmed immunohistochemically by GFP antibody staining. GFP-expressing DP cell lineages were detected in various proliferating cell types of few prenatal fetuses as a proof of DPCs multilineage and the capability to contribute to chimera formation (Fig. 5).

It is well-known that some organs and tissue possess an auto fluorescence that often leads to mistakes when software register a nonspecific or false-positive signal in metabolically active tissue, such as a liver or blood vessels. In order to distinguish GFP-false-positive signals from microscopic artifacts, we analyzed the selected tissue patterns with GFP antibody immunostaining. Hydrogen peroxide 3% was applied to inactivate the cells and tissue that have their own fluorescence.

Fig. 4. Full-growth fetuses and those of arrested development resulted in surgical transfer of blastocysts microinjected with GFP-expressing DPCs. Panels A and B show apparently two fully-developed pups E20.5 obtained with caesarean section from a foster mother. Certain parts of head and body (spine area) of fetuses that, according to our assumption, had cell lineages derived from GFP-DP cells (C, D). It was confirmed with low resolution microscopy (F: GFP image with 488 nm illumination; G: Red= RFP image with 568 nm illumination; H: Green/Red= Alexa Fluor® 647). GFP-expressing cell marker demonstrated at merged image (H). Abnormalities and defects of embryo development due to DPCs microinjection into blastocysts: dead fetuses, arrested development, partially resorbed and extremely small conceptuses as well as multiple sites of encapsulated embryos demonstrated in plates I, J, K, L. Abbreviations: E20.5, prenatal fetuses day 20.5 after mating.

Given the success getting the fetuses after transfer of GFP-DPCs microinjected embryos, we examined tissue patterns immunohistochemically since the GFP-DP cell lineages are represented only in small proportion. Because each glass slide had a counterpart, we had the opportunity to confirm our findings for the patterns that had been previously identified with immunofluorescence as bearing a bright GFP signal. Immunohistochemical analysis confirmed mosaic distribution of GFP-DPCs in all organs and tissue of a few fetuses.

The presence of DP cell lineages was recognized in ectoderm (the brain, spinal cord, and epidermis), endoderm (the liver, lung, heart

muscle and other soft organs of abdominal cavity) and mesoderm (connective tissue, cartilage and bone marrow). GFP expressing DP cell derivatives were found in skin layers and hair bulbs (Fig. 6).

4. DISCUSSION

Earlier experiments in the field of cell reprogramming demonstrate that changes of the differentiation level in specialized epidermal cells can be achieved by nuclear transfer or under the influence of four specific genes regulating cell pluripotency [2,4,18]. There is evidence that induced pluripotent dermal fibroblast can be involved in the formation of teratomas in timus-free mouse [23] and defrosted GFP-MEFs derived iPSCs can participate in organ structures and the tissue building of chimera mouse [24]. Other research demonstrates substantial differences between adult stem-like cells and ESCs after the separation of mixed cell culture into GFP+/GFP- cell populations by flow cytometry and/or using mixed embryonic bodies to transfer under kidney capsules to create teratomas [22].

In this study we made the effort to get evidence that the follicular dermal cells, which are within cell populations from hair bulbs, possess the requisite developmental plasticity to permit pluripotential features. Three separate but related areas of investigation were discovered in current studies: 1- reprogramming or transdifferentiation of DPCs to ES-like cells as a consequence of microinjecting them into blastocysts; 2- capability of DP cells to participate in generation of epiblast egg cylinders *in vitro*; 3- ability microinjected DP cells to survive in a uterus.

Mouse embryos handled from the females as of natural oestrus and those of induced hormonal superovulation were used to microinject with GFP-DPCs. We didn't note any embryonic morphological variation nor any difference of embryo viability or injectability. Assessment showed that approximately 50% of blastocysts did not re-expand after the collapse caused with cell microinjection and no blastocoels could be seen. Some of them did not relieve zona pellucida completely even if trophoblastic cells spread as a layer on the plastic surface in the presence or absence of MEFs. The DP cells penetrating through a zona pellucida allowed blastocysts to hatch but we observed *in vitro* cases of non reversed blastocyst collapse: 56.7% blastocysts in absence of MEFs and 53%

blastocysts cultured on MEFs. Our suggestion that some developmental failures could have arisen due to DPCs microinjection found evidence in the studies *in vivo*.

Fig. 5. Fluorescence microscopy in stained, 8-µm sections of OCT Tissue Tek-embedded foetal tissue derived from microinjected embryos. The antigenicity of the GFP retained in control tissue samples (A-C) of GFP transgenic mice, so that GFP localization became visualized after immunostaining with anti-GFP antibodies. Panels D-U show the fixed and embedded experimental patterns. GFP protein expressing cell lineages were found in bone marrow and connective tissue of body organs (D-F); muscle (G-I); cartilage and connective tissue (J-L); visceral organs (M-O); in head, bone and brain (P-R), and derma (S-U). GREEN = GFP Cells. RED = Autofluorescence. GREEN/RED overlap = Autofluorescence.

To reveal DPCs capability to convert into embryonic-like cells as a result of cell microinjection and detect incorporation of clonal DPCs into blastocysts, we chose to follow *in vivo* experiment conditions. Fluorescent microscopy was used as a specific tool for sorting just those DPCs for microinjection as well as culturing and

transferring only those injected blastocysts which saved bright GFP protein expression [26].

Fig. 6. Hematoxyline/Eosine staining with GFP-antibodies of 8 µm cryosections from OCT embedded fetuses aimed to locate GFP-expressing cells as a proof of chimeric tissue derived from pups carrying DPC lineages. GFP-positive cells were found as DPC lineages in tissues of prenatal pups: GFP antibody labeled these as brown cells in bone marrow, cartilage and connective tissues of a hip bone, single cells in derma (A); thorax bone (B); central cord (C); cartilage tissue around the bone (D), few layers of derma and hair follicles (E, F); head bone and brain tissue (G-I); visceral organs (J), liver (K), trophoblastic and labirinthial layers of placenta (L).

The numerous cases of foetus resorbtion, necrosis and embryonic death were determined, reflecting irreversible events leading to the development arrest. Nevertheless, some part of DP cell population was activated to proliferate but lead to the loss of their slow-cycling. Some DP cells integrated into blastocysts' architecture: 4.5% embryos implanted and 76% foetuses (18 out 24) reached full-time development. Results indicate that GPF-expressing DPCs multiplied and the lineages contributed to derivates from all three germ layers.

In comparison to direct reprogramming, the general problem of the microinjection technique is a gradual loss of acquired features during further prenatal development and keeping just a very small cell population in tissues and organs of the chimaeric offspring. Embryo recovery rate

between control (non-microinjected embryos) and experimental group (microinjected embryos) dramatically decreased; 30% vs 4.5%. The argument exists that the embryonic blastomeres are more viable and can have a suppression effect on injected cells, reducing the contribution of that cells to the epiblast. Microinjected cells also restrain embryonic blastomeres' division and differentiation. Animals belonging to the heterozygous strain could give more prospective opportunity as a source of DP cell subpopulation.

To minimize the possibility of essential changes in gene expression in GFP-DPCs passed through long term culture and subculture (p6-8) but providing a large pool of DPCs, we used earlier passages (p3-4) for *in vivo* trials. We mentioned that DPCs from earlier passage provide better results of embryo survival and that microinjection of bigger cell number is more trustworthy because more visible GFP expression is granted. Due to those simple approaches the main aim of the studies was reached. Microinjected blastocysts were implanted, accomplished gastrulation and organogenesis, and gave the birth to full-time developed offspring.

These results could be used in support of other challenges investigating the role of immune effector mechanisms in the death of chimaeric embryos and the survival of certain types of adult cells in the uterine environment.

5. CONCLUSIONS

We identified hair follicle dermal cells derived from adult mice as a cell type with limited possibility to pluripotency. The cells of follicular dermis from the lower end bulb region of hair follicles, namely dermal papillae cells, can integrate in blastocysts' architecture and participate in formation both as embryonic egg epiblasts and all three germ layers of full-time developed fetuses. Our results provide compelling evidence that clonal DP lines from mouse hair follicle bulbs have extended proliferative capability and can change epigenetic state under the influence of specific embryonic factors and events. They can participate in primary cell differentiation contributing to chimaeric fetuses and give start to the different cell types in various organs and tissues. We also assume that the number of specialized DP cells possessing the features of stem-like cells with high plasticity is boarded but they are able to differentiate into lineages associated not only with the tissues from which they are derived.

The reason for low embryo recovery rate reflecting a low level of implanted blastocysts could be found in the various degrees of cell differentiation, speed of division and epigenetic state for cell types, such as clonal DP cells and embryonic blastomeres.

In this context, the conclusion that our finding is a logical complement to other works in Stem Cell Biology can be made: Injection of Dermal papillae cells into blastocysts and their correspondence with primary embryonic blastomeres can initiate changes in their biological potency and epigenetic state from multipotent to induced pluripotent that are indistinguishable from those of ES cells.

CONSENT

No patients or patients materials were used in this study. It is not applicable.

ETHICAL APPROVAL

The animal protocols used in this work were evaluated and approved by the Animal Use and Ethic Advisory Committee of Durham University, UK. They are in accordance with FELASA Guidelines and The UK's National Committee (The Animal Procedures Committee) for general advice on the operation of the Law and Ethical Review of certain classes of License application (PPL and PIL).

ACKNOWLEDGEMENTS

This work was supported by Heart Research UK grant reference RG2533/07/09. We are grateful to Catherine Kirsanova (EBI, Cambridge) for technical assistance and support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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